1645

INFORMATION DISCLOSURE STATEMENT UNDER 37 C.F.R. 1.56 AND 1.97

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TECH CENTER 1600/2900

In re application of: Parkin and Zierman

For: MEANS AND METHODS FOR MONITORING ANTIRETROVIRAL THERAPY AND GUIDING THERAPEUTIC DECISIONS IN THE TREATMENT OF HIV/AIDS

Attorney Docket No:	11068-0037-999
Serial No:	09/874,472
Filed:	June 4, 2001
Binder:	I of I

A01

Application of: Parkin and Ziermann
Serial No.: 09/874,472
Attorney Docket No.: 11068-0037-999

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

To:
JOHN P. WHITE
COOPER & DUNHAM LLP
1185 AVENUE OF THE AMERICAS
NEW YORK, NY 10036

28 2003

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

Applicant's or agent's file reference 59597-E-PCT	Date of Mailing (*day/month/year*) 23 JAN 2003 **FOR FURTHER ACTION** See paragraphs 1 and 4 below
International application No. PCT/US02/18684	International filing date (*day/month/year*) 04 June 2002 (04.06.2002)
Applicant VIROLOGIC, INC.	

1. ☐ The applicant is hereby notified that the international search report has been established and is transmitted herewith.

   Filing of amendments and statement under Article 19: 2 MO ART 19 DUE. 3.23.03 - AP
   The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

   When? The time limit for filing such amendments is normally two months from the date of transmittal of the international search report.

   Where? Directly to the International Bureau of WIPO, 34, chemin des Colombettes 3 MO IDS DUE 4.23.03
   1211 Geneva 20, Switzerland, Facsimile No.: (41-22) 740.14.35

   For more detailed instructions, see the notes on the accompanying sheet. (59597-E, 59597-D, 59597-C, 59597-B, 59597-A, - AP

2. ☐ The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. ☐ **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

   ☐ the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

   ☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Reminders**

   Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90 *bis*.1 and 90 *bis*.3, respectively, before the completion of the technical preparations for international publication. 12.4.03 - AP

   Within **19 months** from the priority date, but only in respect of some designated Offices, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase **until 30 months** from the priority date (in some Offices even later); otherwise the applicant must, **within 20 months** from the priority date, perform the prescribed acts for entry into the national phase before those designated Offices. 1.4.03 - AP 2.4.03 - AP

   In respect of other designated Offices, the time limit of **30 months** (or later) will apply even if no demand is filed within 19 months.

   See the Annex to Form PCT/IB/301 and, for details about the applicable time limits, Office by Office, see the *PCT Applicant's Guide*, Volume II, National Chapters and the WIPO Internet site.

Name and mailing address of the ISA/US Commissioner for Patents Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Shannon Foley Telephone No. (703) 308-0196

Form PCT/ISA/220 (April 2002) *(See notes on accompanying sheet)*

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 59597-E-PCT/	**FOR FURTHER ACTION**	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
International application No. PCT/US02/18684	International filing date (*day/month/year*) 04 June 2002 (04.06.2002)	(Earliest) Priority Date (*day/month/year*) 04 June 2001 (04.06.2001)
Applicant VIROLOGIC, INC.		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 60 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the Report**
   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.
   ☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).
   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing:
   ☐ contained in the international application in written form.
   ☐ filed together with the international application in computer readable form.
   ☐ furnished subsequently to this Authority in written form.
   ☐ furnished subsequently to this Authority in computer readable form.
   ☐ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   ☐ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.
2. ☐ **Certain claims were found unsearchable** (See Box I).
3. ☒ **Unity of invention is lacking** (See Box II).
4. With regard to the **title**,
   ☒ the text is approved as submitted by the applicant.
   ☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,
   ☒ the text is approved as submitted by the applicant.
   ☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. ____
   ☐ as suggested by the applicant. ☒ None of the figures
   ☐ because the applicant failed to suggest a figure.
   ☐ because this figure better characterizes the invention.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/18684

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-12

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/18684

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : C12Q 1/18, 1/68, 1/70
US CL : 435/5, 6, 32
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/5, 6, 32

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZIERMANN et al. A mutation in human immunodeficiency virus type 1 protease, N88S, that causes in vitro hypersensitivity to amprenavir. J. Virology. May 2000. Vol 74. No. 9, pages 4414-4419, especially pages 4415-4416.	1-12

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
12 December 2002 (12.12.2002)

Date of mailing of the international search report
23 JAN 2003

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703)305-3230

Authorized officer
Shanon Foley
Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

PCT/US02/18684

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-12, drawn to a method of assessing the effectiveness of protease antiretroviral therapy in an HIV-infected subject by evaluating the presence of a mutation at codon 88.

Group II, claim(s) 13-16, drawn to a method of evaluating the effectiveness of an HIV antiretroviral drug candidate with a vector encoding a mutation at codon 88.

Group III, claim(s) 17-20, drawn to a resistance test vector comprising an HIV patient-derived segment having a mutation at codon 88.

Group IV, claim(s) 21, drawn to a method for evaluating the viral fitness of a patient's virus.

Group V, claim(s) 22-44, 80-85, 98-113, 117-120, drawn to a method of evaluating the effectiveness of an HIV antiretroviral drug candidate with a vector encoding a mutation at codon 82.

Group VI, claim(s) 45-67, 80-85, 98-120, drawn to a method of evaluating the effectiveness of an HIV antiretroviral drug candidate with a vector encoding a mutation at codon 90.

Group VII, claim(s) 68-70, drawn to a method of evaluating the effectiveness of an HIV antiretroviral drug candidate with a vector encoding a mutation at codon 82 and 90.

Group VIII, claim(s) 71, 72, and 86, drawn to a method of evaluating the effectiveness of an HIV antiretroviral drug candidate with a vector encoding a mutation at codon 82.

Group IX, claim(s) 73, 74, and 86, drawn to a method of evaluating the effectiveness of an HIV antiretroviral drug candidate with a vector encoding a mutation at codon 90.

Group X, claim(s) 74 and 75, drawn to a method of evaluating the effectiveness of an HIV antiretroviral drug candidate with a vector encoding a mutation at codon 82 and 90.

Group XI, claim(s) 76, 77, 87-91, 121, and 122, drawn to a resistance test vector comprising an HIV patient-derived segment having a mutation at codon 82.

Group XII, claim(s) 78, 87-91, 121, and 122, drawn to a resistance test vector comprising an HIV patient-derived segment having a mutation at codon 90.

Group XIII, claim(s) 79 and 92-97, drawn to a method for determining the replication capacity for a patient's virus.

Group XIV, claim(s) 123 and 125, drawn to a method for determining whether a patient's virus, comprising a mutation at codon 30, is resistant to protease inhibitor drugs.

Group XV, claim(s) 124, drawn to drawn to a method for determining whether a patient's virus is resistant to protease inhibitor drugs.

Group XVI, claim(s) 126, drawn to drawn to a method for determining whether a patient's virus, comprising a mutation at codon 50, is resistant to protease inhibitor drugs.

INTERNATIONAL SEARCH REPORT

PCT/US02/18684

The inventions listed as Groups I-XVI do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The special technical feature of group I is drawn to a method of assessing the effectiveness of an antiretroviral therapy by evaluating whether an HIV sample comprises a mutation at codon 88. Any subsequent group that does not share this special technical feature lacks unity of invention with the first group.

The special technical feature if group II is drawn to a method of evaluating the effectiveness of an HIV antiretroviral drug candidate with a vector encoding a mutation at codon 88. This group does not share the special technical feature with group I because the groups comprise different method steps and ingredients.

The special technical feature if group III is drawn to a first product, a vector encoding a mutation at codon 88. This group does not share the special technical feature with group I because the product is not required to practice the method of group I.

The special technical feature of group IV is a method of evaluating the viral fitness of a patient's virus. This group does not share the special technical feature with group I because the method steps are different from the method of group I and requires different ingredients.

The special technical feature of group V is drawn to a method of assessing the effectiveness of an antiretroviral therapy by evaluating whether an HIV sample comprises a mutation at codon 82. This group does not share the special technical feature with group I because the method steps are drawn to evaluating a different sequence that distinguishes the special technical feature in group I.

The special technical feature of group VI is drawn to a method of assessing the effectiveness of an antiretroviral therapy by evaluating whether an HIV sample comprises a mutation at codon 90. This group does not share the special technical feature with group I because the method steps are drawn to evaluating a different sequence that distinguishes the special technical feature in group I.

The special technical feature of group VII is drawn to a method of assessing the effectiveness of an antiretroviral therapy by evaluating whether an HIV sample comprises a mutation at codon 82 and 90. This group does not share the special technical feature with group I because the method steps are drawn to evaluating a different sequence that distinguishes the special technical feature in group I.

The special technical feature if group VIII is drawn to a method of evaluating the effectiveness of an HIV antiretroviral drug candidate with a vector encoding a mutation at codon 82. This group does not share the special technical feature with group I because the groups comprise different method steps and ingredients.

The special technical feature if group IX is drawn to a method of evaluating the effectiveness of an HIV antiretroviral drug candidate with a vector encoding a mutation at codon 90. This group does not share the special technical feature with group I because the groups comprise different method steps and ingredients.

The special technical feature if group X is drawn to a method of evaluating the effectiveness of an HIV antiretroviral drug candidate with a vector encoding a mutation at codon 82 and 90. This group does not share the special technical feature with group I because the groups comprise different method steps and ingredients.

The special technical feature if group XI is drawn to a second product, a vector encoding a mutation at codon 82. This group does not share the special technical feature with group I because the product is not required to practice the method of group I.

The special technical feature if group XII is drawn to a third product, a vector encoding a mutation at codon 90. This group does not share the special technical feature with group I because the product is not required to practice the method of group I.

The special technical feature of group XIII is a method for determining the replication capacity of a patient's virus. This group does not share the special technical feature with group I because the method steps are different from the method of group I and requires different ingredients.

The special technical feature of group XIV is drawn to a method of determining whether an HIV virus is resistant to a protease inhibitor drug by determining whether the sample has a mutation at codon 30 exists. This group does not share the special technical feature with group I because the method steps and ingredients to practice each of the methods is distinctly different.

The special technical feature of group XV is drawn to a method of determining whether an HIV virus is resistant to a protease inhibitor drug by determining whether the sample is resistant to any one protease inhibitor drug. This group does not share the special technical feature with group I because the method steps and ingredients to practice each of the methods is distinctly different.

The special technical feature of group XVI is drawn to a method of determining whether an HIV virus is resistant to a protease inhibitor drug by determining whether the sample has a mutation at codon 50 exists. This group does not share the special technical feature with group I because the method steps and ingredients to practice each of the methods is distinctly different.

Only claims 1-12 will be searched if applicant does not agree to pay for any additional groups.

INTERNATIONAL SEARCH REPORT

PCT/US02/18684

Continuation of B. FIELDS SEARCHED Item 3:
USPatfull, USPGpub, EPO, JPO, Derwent, medline, embase, biosis
search terms: amprenavir, 88, resist, codon, HIV, mutat

NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under Article 19. The Notes are based on the requirements of the Patent Cooperation Treaty and of the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule" and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions, respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended ?

The claims only.

The description and the drawings may only be amended during international preliminary examination under Chapter II.

When ? **Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).**

Where not to file the amendments ?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How ? **Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.**

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

What documents must/may accompany the amendments ?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confounded with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped), whether

(i) the claim is unchanged;
(ii) the claim is cancelled;
(iii) the claim is new;
(iv) the claim replaces one or more claims as filed;
(v) the claim is the result of the division of a claim as filed.

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

To:
JOHN P. WHITE
COOPER & DUNHAM LLP
1185 AVENUE OF THE AMERICAS
NEW YORK, NY 10036

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

	Date of Mailing (*day/month/year*) 23 JAN 2003
Applicant's or agent's file reference 59597-E-PCT/	**FOR FURTHER ACTION** See paragraphs 1 and 4 below
International application No. PCT/US02/18684	International filing date (*day/month/year*) 04 June 2002 (04.06.2002)
Applicant VIROLOGIC, INC.	

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   The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

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   Where? Directly to the International Bureau of WIPO, 34, chemin des Colombettes 1211 Geneva 20, Switzerland, Facsimile No.: (41-22) 740.14.35

   For more detailed instructions, see the notes on the accompanying sheet.

2. ☐ The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

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   In respect of other designated Offices, the time limit of **30 months** (or later) will apply even if no demand is filed within 19 months.

   See the Annex to Form PCT/IB/301 and, for details about the applicable time limits, Office by Office, see the *PCT Applicant's Guide*, Volume II, National Chapters and the WIPO Internet site.

Name and mailing address of the ISA/US Commissioner for Patents Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Shannon Foley Telephone No. (703) 308-0196

Form PCT/ISA/220 (April 2002) *(See notes on accompanying sheet)*

A02

Application of: Parkin and Ziermann
Serial No.: 09/874,472
Attorney Docket No.: 11068-0037-999

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Vol. 74, No. 9

NOTES

A Mutation in Human Immunodeficiency Virus Type 1 Protease, N88S, That Causes In Vitro Hypersensitivity to Amprenavir

RAINER ZIERMANN,[1] KAY LIMOLI,[1] KALYAN DAS,[2] EDWARD ARNOLD,[2] CHRISTOS J. PETROPOULOS,[1] AND NEIL T. PARKIN[1]*

ViroLogic Inc., South San Francisco, California 94080,[1] and Center for Advanced Biotechnology and Medicine (CABM) and Rutgers University Chemistry Department, Piscataway, New Jersey 08854-5638[2]

Received 25 October 1999/Accepted 18 January 2000

Amprenavir (Agenerase, 141-W94, VX-478) is a human immunodeficiency virus type 1 (HIV-1) protease inhibitor (PRI) recently approved for the treatment of HIV-1 infection in the United States. A major cause of treatment failure is the development of resistance to PRIs. One potential use for amprenavir is as salvage therapy for patients for whom treatment that includes one (or more) of the other four currently approved PRIs—saquinavir, indinavir, ritonavir, and nelfinavir—has failed. We evaluated the cross-resistance to amprenavir of viruses that evolved during treatment with the two most commonly prescribed PRIs, nelfinavir and indinavir. Unexpectedly, a dramatic increase in susceptibility (2.5- to 12.5-fold) was observed with 20 of 312 (6.4%) patient viruses analyzed. The most pronounced increases in susceptibility were strongly associated with an N88S mutation in protease. All viruses that carried the N88S mutation were hypersensitive to amprenavir. Site-directed mutagenesis studies confirmed the causal role of N88S in determining amprenavir hypersensitivity. The presence of the N88S mutation and associated amprenavir hypersensitivity may be useful in predicting an improved clinical response to amprenavir salvage therapy.

Since the discovery of human immunodeficiency virus (HIV) in the early 1980s, an intense drug discovery effort has resulted in the approval of 14 antiviral drugs, with many more in development (3). The approved drugs inhibit HIV replication by interfering with the enzymatic activities of either protease (PR) or reverse transcriptase (RT). The lack of proofreading functions that is inherent in RT, coupled with error-prone replication at a high rate, allows HIV to mutate readily. This high mutation frequency contributes to the ability of HIV to evade successful long-term therapy, resulting in viral load rebound (4).

Multi-drug-resistant HIV variants pose an increasing problem for the care of infected patients (2, 5, 6, 10). Determination of the genotypes of such viruses frequently reveals complex patterns of mutations in PR and RT, thus complicating predictions of sensitivity or resistance to antiviral drugs. Accurate measurements of phenotypic drug susceptibility can be used to determine patterns of cross-resistance to existing drugs. This type of analysis is an increasingly important tool for evaluating investigational or newly approved drugs. The most recently approved PR inhibitor (PRI), amprenavir, is a promising candidate for salvage therapies, in part because available data suggest good activity against viruses resistant to other PRIs (12, 13, 21).

Utilizing a recently described phenotypic drug-susceptibility assay (14, 16a), we determined the drug susceptibilities of viruses present in infected individuals whose combination therapy regimens, including nelfinavir or indinavir, the two most commonly prescribed PRIs, were failing (viral loads of >500 copies per ml). A measure of relative susceptibility is obtained by comparing the 50% inhibitory concentration (IC_{50}) for the patient virus to that for a reference virus, derived from the NL4-3 infectious HIV type 1 (HIV-1) DNA clone (1). Values obtained from single measurements that are less than or more than 2.5-fold those of the reference exceed the normal variation of the assay and are considered indicative of altered susceptibility to a drug. Hypersensitivity is defined as a reduction in the IC_{50} of ≥2.5-fold (i.e., a susceptibility value of ≤0.4) relative to the NL4-3 reference strain. A significant proportion of viruses (20 of 312, or 6.4%) from patients that had been treated with nelfinavir and/or indinavir exhibited hypersensitivity to amprenavir (Table 1).

Table 1 lists drug susceptibility data for 20 patient-derived viruses, in order of decreasing susceptibility to amprenavir. All but one of the 20 viruses (patient 17) exhibited decreased susceptibility to at least one PRI, most frequently nelfinavir. The PR genotypes of the viruses displaying hypersensitivity to amprenavir were determined by DNA sequencing using conventional dideoxynucleotide chain terminating sequencing methods (Perkin-Elmer Biosystems, Foster City, Calif.). Deduced PR amino acid sequences were compared to that of NL4-3 (Table 1) and to a list of mutations associated with PRI resistance (19). Thirteen of the 20 patient virus populations (65%) and notably all of the viruses displaying the most pronounced increases in susceptibility (≤0.2 relative susceptibility, which corresponds to a ≥5-fold increase in susceptibility) had an asparagine (N) to serine (S) substitution in PR at amino acid position 88. N88S was always observed in combination with mutations at various other positions, including amino acids 20, 36, 46, 63, and 77. No viruses from drug-naïve patients

* Corresponding author. Mailing address: 270 East Grand Ave., South San Francisco, CA 94080. Phone: (650) 866-7438. Fax: (650) 742-0993. E-mail: nparkin@virologic.com.

TABLE 1. PRI susceptibilities and PR genotypes of 20 patient-derived HIV-1 viruses displaying hypersensitivity to amprenavir

Patient no.	Relative susceptibility[a]					PR mutation[b]	
	SQV	IDV	RTV	NFV	AMP	Resistance associated	Polymorphism(s)
1	0.73	**2.11**	1.72	**8.92**	**0.08**	K20T, M36I, L63Q, N88S	K14R, I15V, E35D, R41K, I62V
2	**0.26**	**6.16**	1.50	**21.06**	**0.09**	M46L, L63P, N88S	I13I/V, E35D, I64V, I72V
3	1.55	**3.15**	1.22	**11.06**	**0.10**	L63P, V77I, N88S	I62V
4	1.20	1.49	**3.38**	**15.87**	**0.15**	M36I, L63P, A71A/T, N88S	I13V, E35D, I62V
5	1.88	**6.31**	1.49	**29.95**	**0.15**	K20M, M36V, M46I, L63P, N88S	I13V, N37A, I62V, I93L
6	1.41	**5.47**	1.85	**16.76**	**0.16**	M46I, L63P, V77I, N88S	I93I/L
7	1.28	**7.61**	**3.36**	**24.67**	**0.16**	M46I, L63P, N88S	I13V, K14R, N37D, I93L
8	1.80	**7.56**	1.95	**18.61**	**0.20**	M46I, L63P, V77I, N88S	I13V, R41K, I93L
9	1.81	1.15	**3.70**	5.71	**0.23**	L10I, K20K/R, M36I, I54V, L63H, L90L/M	I13V, I62V, I64V, I72V, N83D
10	**2.05**	**5.58**	1.59	**15.18**	**0.24**	L10I, M46I, L63P, A71T, V77I, N88S	E35D, N37S
11	1.22	**4.55**	**2.55**	**9.55**	**0.28**	L63P, V77I, N88N/S	R41K, I62V, I93I/L
12	**0.12**	0.77	**3.81**	1.24	**0.29**	L10L/F, M36M/L, M46M/I, L63S, V77I, V82A/T	I64V, I72T
13	**0.38**	1.06	**8.12**	1.65	**0.30**	K20R, M36I, I54V, V82A	R41K, I64V, H69Y
14	**8.99**	**13.59**	**6.29**	**63.05**	**0.30**	K20T, L63P, A71V, N88S, L90M	I15V, R41K, K45R, R57K, I72M
15	0.54	1.27	0.83	**2.59**	**0.32**	K20K/M, L63P, A71A/T, N88N/D/S	I13V, I15V, E35D, N37D
16	0.62	1.12	**4.36**	**3.56**	**0.32**	M36I, I54I/V, L63P, A71A/V, V82V/A	E35D, I62V, H69Q
17	0.53	0.60	0.57	**0.45**	**0.33**	M36M/I, L63Q, V77V/I, N88N/S	K14R, I15V, N37N/S, R41K, I62V
18	**0.42**	1.28	0.63	**27.62**	**0.36**	D30N, M36I, L63H, A71V	I13V, E35D, N37D, R57K, I62V, I64I/V, I93L
19	**0.32**	0.69	**0.35**	**2.99**	**0.37**	D30D/N, M36V	E35D, N37S, I64V
20	1.16	1.37	1.15	**29.13**	**0.37**	D30N, L33I, M36M/I, L63P, N88D	T12A/V, I13V, R57K, I93L

[a] Values represent the fold change in IC_{50} relative to the reference, representing the mean of the two determinations. Reproducibility studies have demonstrated the variability of PRI drug susceptibility results to be less than twofold with replicate testing of samples. Mean relative susceptibility values (the IC_{50} for virus from the patient divided by the IC_{50} for the reference) of >2, indicative of reduced susceptibility, or of <0.5, indicative of increased susceptibility, are highlighted in bold type. Values are rounded to two decimal places. SQV, saquinavir; IDV, indinavir; RTV, ritonavir; NFV, nelfinavir; AMP, amprenavir.

[b] Mixed virus populations exhibiting more than one amino acid at given positions are indicated by slashes.

were found to carry this substitution (2, 10; our unpublished data). While other changes at amino acid 88, most commonly N88D, occurred in viruses isolated from patients for whom treatment with PRIs was failing, these alterations are not correlated with a significant increase in susceptibility to amprenavir (data not shown). Mutations D30N and V82A, each found in 3 of the 20 patient-derived viruses listed in Table 1, were less frequently associated with amprenavir hypersensitivity. However, the increases in amprenavir susceptibility were less dramatic than those with N88S. These substitutions were also observed in viruses that did not exhibit hypersensitivity to amprenavir (data not shown), implying less direct roles for D30N and V82A in increased amprenavir susceptibility.

A representative PRI susceptibility profile for virus from patient 1 is shown in Fig. 1. The shift of the amprenavir susceptibility curve to lower drug concentrations, i.e., to the left of the reference curve, reflects increased susceptibility. The nelfinavir curve is shifted towards higher drug concentrations, i.e., to the right, indicating reduced susceptibility. Susceptibilities to saquinavir, indinavir, and ritonavir did not vary significantly (less than 2.5-fold) from those of the reference strain.

Based on the available treatment histories of the patients harboring viruses with hypersensitivity to amprenavir, this phenotype appears to be associated with the failure of nelfinavir- or indinavir-containing treatment regimens. N88S has been identified previously in viruses isolated after in vitro passage in the presence of an investigational PRI, SC-55389A (20, 22), and in viruses isolated from patients treated with nelfinavir (16). Recently, viruses which emerged early after in vitro selection in the presence of an investigational PRI, BMS 232632, were found to contain the N88S mutation (4a). Intriguingly, N88S was found to arise in only two of the three laboratory strains used, suggesting that different genetic backgrounds could predispose viruses to alternative resistance pathways of decreased PRI susceptibility. These data raise the possibility that new PRIs may select more frequently for the N88S substitution than do nelfinavir or indinavir.

To delineate the role of individual mutations in PRI susceptibility, site-specific oligonucleotide-directed mutagenesis was used to generate a series of virus mutants. Mutations were introduced into the PR coding region of the NL4-3-derived reference vector using a PCR "megaprimer" method (18). The presence of the desired mutation(s) and absence of other substitutions was confirmed by DNA sequencing. Oligonucleotide RsrII (5′-ACTTTCGGACCGTCCATTCCTGGCTTTAATT TTACTGGTACAG-3′) was used to introduce a unique *Rsr*II restriction site (underlined) approximately 50 nucleotides downstream of the PR coding region into our drug-sensitive reference vector. The three point mutations introduced (bold) do not change the coding sequence of RT. The resulting construct allows an exchange of the PR coding region using a 590-bp *Apa*I-*Rsr*II fragment, since both restriction sites are unique. All subsequent mutants were constructed by exchanging *Apa*I-*Rsr*II fragments. The following oligonucleotides were used to introduce specific point mutations (bold type): K20T (5′-GGGGGGCAATTAACGGAAGCTCTATTAG-3′; coding strand), M36I (5′-GTATTAGAAGAAATAAATTTGCC AGGAAG-3′; coding strand), M46L (5′-GATGGAAACCAA AATTGATAGGGGGAATTG-3′; coding strand), L63P (5′-G TATGATCAGATACCCATAGAAATCTGC-3′; coding strand), N88S (5′-CTGAGTCAACAGACTTCTTCCAATTATG-3′; noncoding strand). Phenotypic analysis was performed on a total of 12 mutants (Table 2). Representative susceptibility curves for nelfinavir, indinavir, and amprenavir for mutant viruses carrying N88S and L63P/V77I/N88S are shown in Fig. 2. The N88S mutant and all other viable mutants that carried N88S were hypersensitive to amprenavir, with relative susceptibility values ranging from 0.04 to 0.14. Mutants containing N88S also displayed reduced susceptibility to nelfinavir (2.39- to 12.89-fold). Mutant viruses containing L63P or L63P/V77I

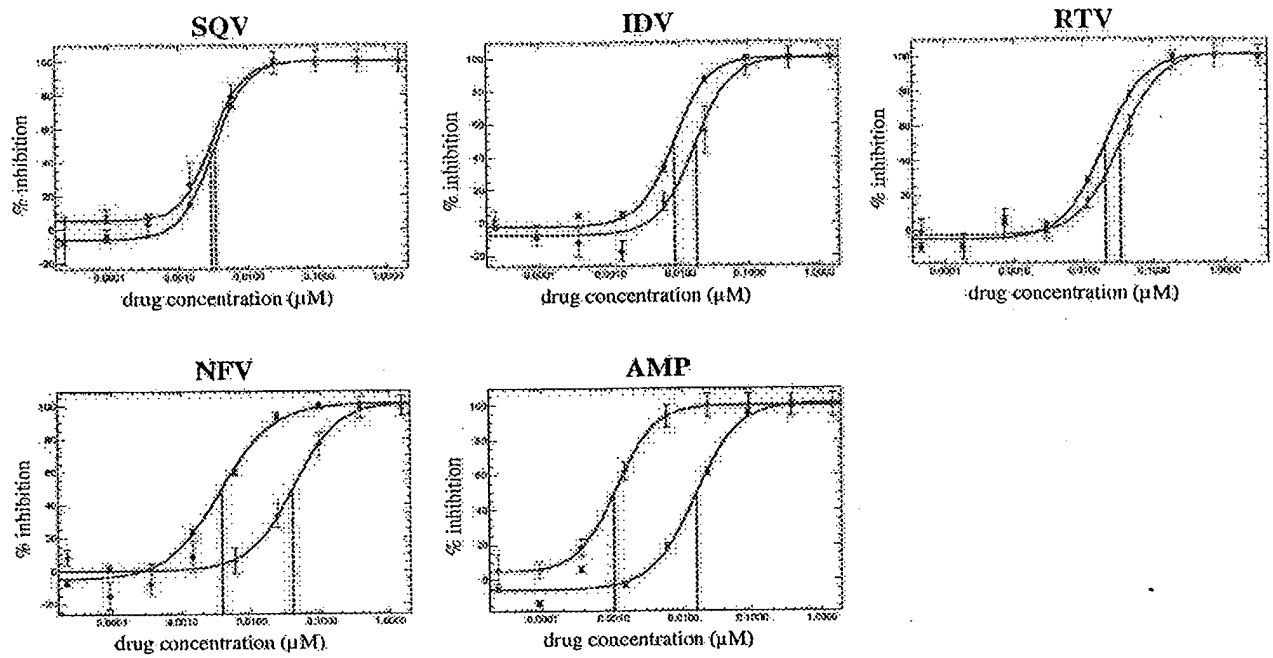

FIG. 1. PRI susceptibility profile of virus from patient 1. Susceptibility to PRI drugs was assessed by comparing drug susceptibility curves obtained using the vector containing PR and RT coding sequences from the patient-derived virus (red lines) with a reference construct containing PR and RT from pNL4-3 (blue lines). The IC_{50} is determined from the curve and indicated by vertical lines. The IC_{50}s of saquinavir (SQV), indinavir (IDV), ritonavir (RTV), nelfinavir (NFV), and amprenavir (AMP) for the reference control were 3.4, 8.3, 20.6, 3.8, and 16.0 nM, respectively.

alone were not hypersensitive to amprenavir, thus confirming that the N88S mutation is sufficient to determine this phenotype. Clearly, M46L, L63P, and V77I each play a role in decreased susceptibility to indinavir and nelfinavir when present in combination with N88S. The L63P/V77I double mutant exhibited a small, 2.49-fold reduction in nelfinavir susceptibility. The K20T virus displayed increased susceptibility to all five PRIs, with values ranging from 0.37 to 0.47. No change in susceptibility to any of the nine approved RT inhibitors was found (data not shown).

In the absence of drug, the N88S mutant produced significantly less luciferase activity than the parental reference virus

TABLE 2. Susceptibility to PRIs and relative luciferase activity of HIV-1 mutants

Site-directed PR mutation(s)	Relative susceptibility[a]					Relative luciferase activity[b]
	SQV	IDV	RTV	NFV	AMP	
N88S	**0.47**	1.56	**0.36**	**2.39**	**0.04**	1.0
L63P, N88S	1.44	**2.56**	0.77	**5.10**	**0.11**	20.7
M46L, L63P, N88S	1.15	**2.30**	0.85	**8.18**	**0.12**	28.0
L63P, V77I, N88S	1.24	**3.09**	1.39	**12.89**	**0.08**	29.3
M46L, L63P, V77I, N88S	1.45	**2.97**	1.33	**12.24**	**0.14**	53.2
K20T	**0.37**	**0.47**	**0.47**	**0.43**	**0.38**	10.9
L63P	1.04	1.12	1.27	1.43	1.06	163.9
L63P, V77I	1.24	1.72	1.73	**2.49**	0.91	75.6
K20T, N88S			Not viable			<0.01
K20T, L63P, N88S			Not viable			<0.01
K20T, M36I, L63P, N88S			Not viable			<0.01
K20T, M46L, L63P, N88S			Not viable			<0.01

[a] Multiple clones that contained the indicated mutations were tested for PRI susceptibility individually (two to five independent clones each), and the mean relative susceptibilities were determined. See Table 1, footnote *a*, for PRI abbreviations.

[b] The mean relative luciferase activity (expressed as a percentage of that of the reference) is defined as relative light units produced by the PR mutant divided by relative light units produced by the reference virus, in the absence of a drug.

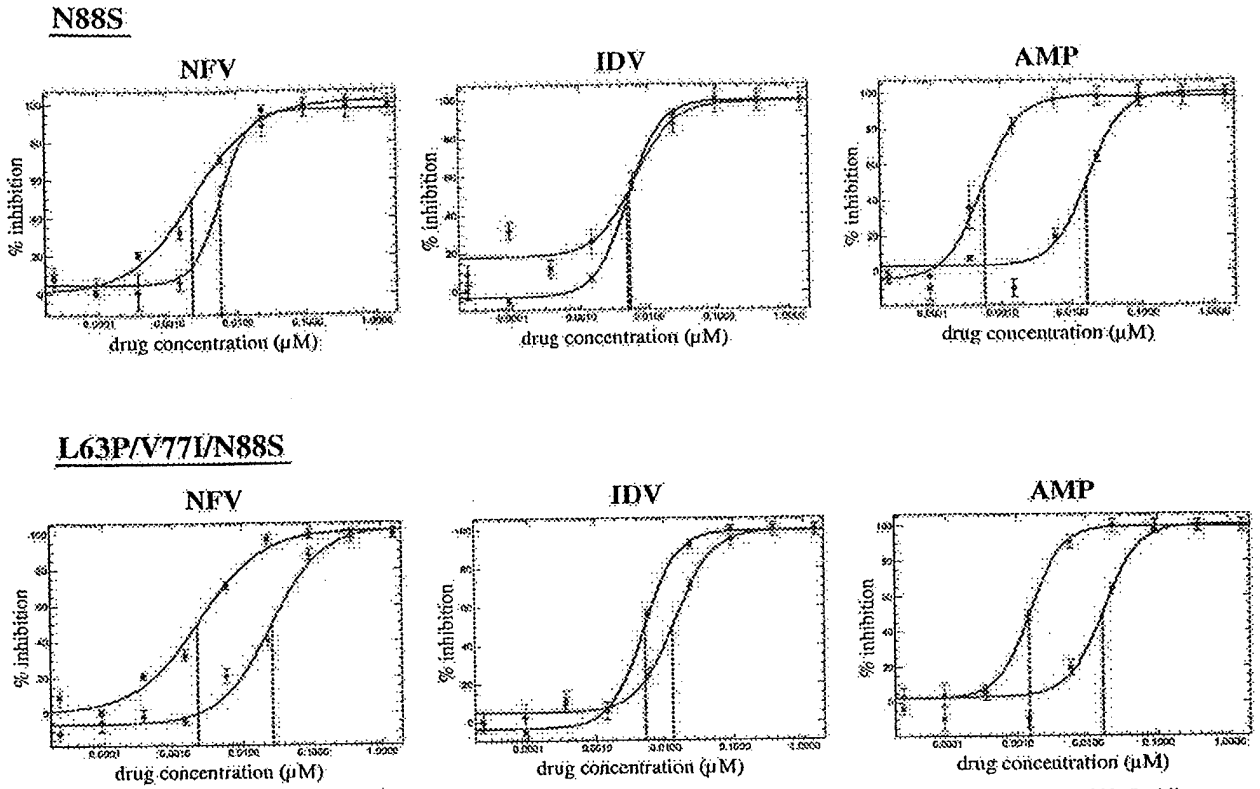

FIG. 2. NFV, IDV, and AMP susceptibility curves for two representative mutants with site-directed mutations, N88S and L63P/V77I/N88S. Red lines represent respective mutant constructs; blue lines represent the reference construct. For drug abbreviations, see the legend for Fig. 1.

(~1%). A virus containing both mutations K20T and N88S could not be analyzed, since it did not produce sufficient luciferase activity. The introduction of additional substitutions at position 63, positions 36 and 63, or positions 46 and 63 to K20T/N88S mutants did not restore luciferase activity. To ensure that the K20T/N88S mutants did not carry other mutations that might result in inactive constructs, the *Apa*I-*Rsr*II restriction fragment covering all of the HIV PR of the K20T/N88S mutant was exchanged with the equivalent fragment of the M46L/L63P/V77I/N88S mutant. Luciferase activity was restored, and phenotypic data were consistent with the results presented in Table 2 (data not shown). Thus, the vector backbone used to generate all mutants harboring K20T/N88S is functional.

Viruses derived from patients 1 and 14 carried both K20T and N88S substitutions and generated modest amounts of luciferase activity, although the activity was markedly less than that of the control (data not shown). One attractive model that may explain these observations is that these mutants are severely fitness impaired. The luciferase activity measured in the absence of a drug may be interpreted as an indicator of how well the recombinant virus pool is able to replicate. Luciferase activity may serve as a potential measure of replicative fitness for the following reasons: (i) given equal transfection efficiencies with the same test vector DNA pool, relative luciferase activity is highly reproducible; (ii) relative luciferase activity values obtained with different viral constructs vary; and (iii) most, though not all, virus mutants generate lower relative luciferase activity values than the drug-sensitive reference virus (our unpublished observations). This is in accordance with the general idea that the fitness of most drug-resistant HIV variants is less than that of wild-type HIV. Recombinant viruses containing N88S have been reported to display delayed growth kinetics compared to parental wild-type HIV-1 (20, 22), adding support to this concept. If this is correct, it is likely that additional mutations, generally referred to as polymorphisms (e.g., I15V and R41K), enhance the fitness of patient 1- or 14-derived virus compared to that of the K20T/N88S site-directed mutant. Experiments to shed light on these issues and to explore the suitability of this single-replication-cycle assay to evaluate viral fitness are currently under way.

It is relevant to determine how the amprenavir concentrations used in the phenotypic assay correlate with plasma drug levels in patients undergoing therapy. The IC_{50}s and IC_{95}s for the NL4-3 reference strain and the amprenavir-hypersensitive patient strains were compared to the estimated trough concentrations of amprenavir in plasma (17). Amprenavir has an IC_{50} of 8 to 16 nM and an IC_{95} about 10 times higher (100 to 139 nM) for the NL4-3 reference strain. Similar IC_{50}s and IC_{95}s for wild-type strains have been reported by other investigators using different assays (9, 13, 15, 21). Values for N88S mutant viruses ranged from 0.9 to 5.8 nM (IC_{50}) and 7 to 70 nM (IC_{95}). The concentration of amprenavir in plasma 12 h after a dose of 1,200 mg is approximately 0.65 μg/ml, or 1,300 nM (17). Since 90% of circulating amprenavir is bound to plasma proteins (11), the effective minimum concentration is 130 nM. Thus, the range in IC_{95}s for N88S-containing viruses is below the amprenavir trough level, whereas that for wild-type viruses some-

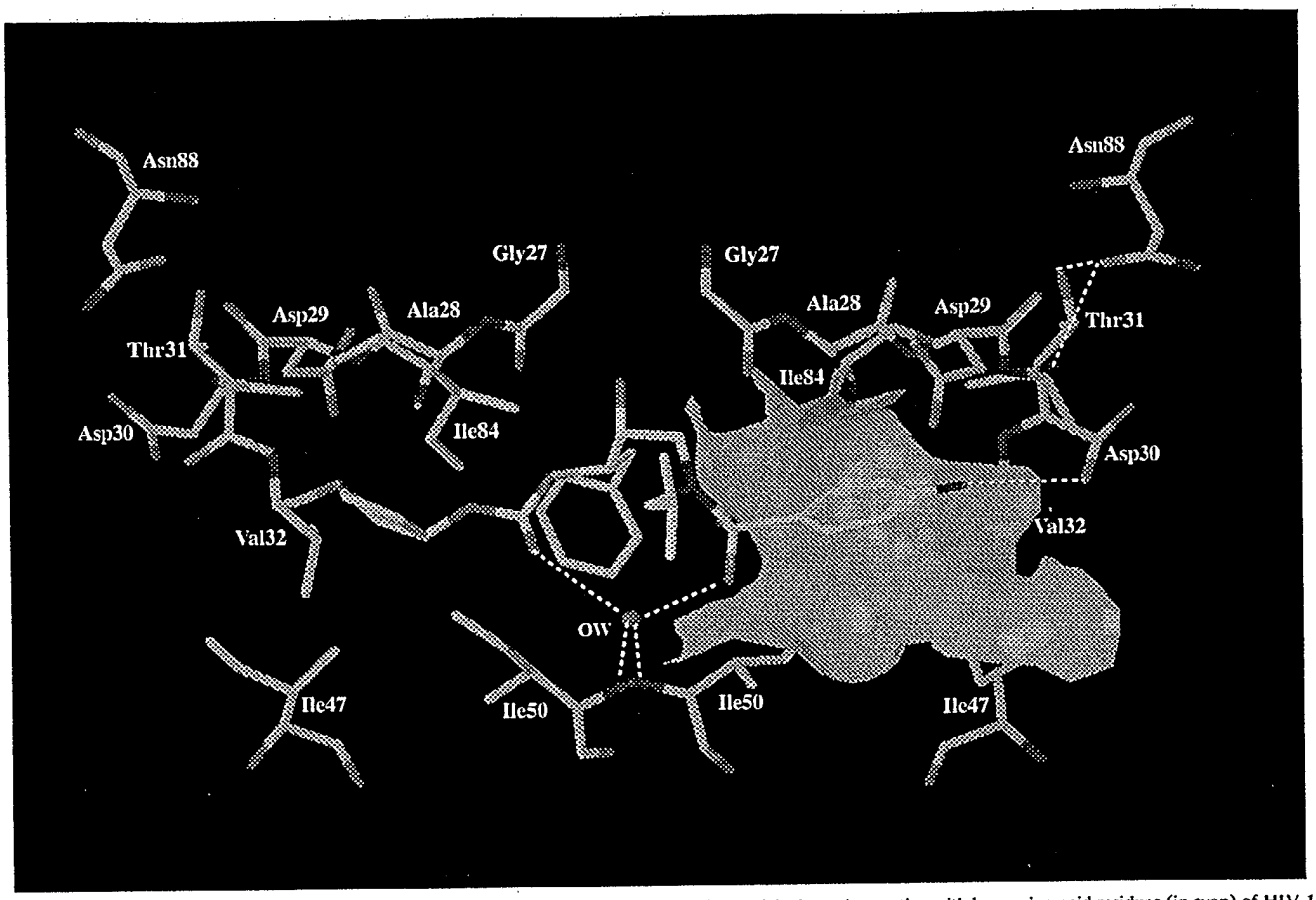

FIG. 3. Postulated interactions of amprenavir bound to HIV-1 protease. Amprenavir (in gray) is shown interacting with key amino acid residues (in cyan) of HIV-1 PR in the crystal structure of HIV-1 PR complexed with amprenavir (7). Nitrogen and oxygen atoms are colored blue and red, respectively, and OW represents a key conserved water molecule in the pocket. The molecular surface is calculated using the side-chain atoms of A28, V32, I47, I50, and I84 and Cα of A28. The figure was drawn using the program SYBYL, version 6.3 (Tripos, Inc.).

times exceeds it. The decreases in amprenavir IC_{50} and IC_{95} observed for viruses carrying the N88S mutation in PR therefore appear to be potentially clinically relevant, since such viruses are less likely to escape the suppressive effects of the drug than are wild-type viruses with higher IC_{95} values. When other variables that negatively affect drug trough levels are considered, such as alterations in levels of amprenavir binding proteins (in particular α1-acid glycoprotein) or brief interruptions in drug dosing, the effect of N88S on the viral response to amprenavir could be even more important.

All of the N88S viruses we analyzed had increased susceptibility to amprenavir. N88S mutant viruses emerge in patients undergoing nelfinavir and/or indinavir treatment, and this mutation has been linked to decreased susceptibility to both drugs. This finding is reminiscent of the increase in susceptibility to zidovudine and adefovir caused by the M184V mutation in RT, which confers high-level resistance to 3TC (8; H. Tian et al., Abstr. 2nd Int. Workshop HIV Drug Resistance Treatment Strategies, abstr. 30, 1998; M. Miller et al., Abstr. 2nd Int. Workshop HIV Drug Resistance Treatment Strategies, abstr. 34, 1998). However, M184V severely decreases susceptibility to 3TC, while the N88S substitution, in combination with other mutations, causes a relatively moderate decrease in susceptibility to nelfinavir and indinavir. N88S alone marginally affects susceptibility to nelfinavir (Table 2). Therefore, in contrast to M184V, which is readily selected by 3TC treatment, N88S is less likely to arise as a primary resistance mutation in response to nelfinavir or indinavir therapy. This notion is supported by the finding that viruses containing the N88S substitution alone have not been isolated from patients, suggesting that N88S may be selected primarily in viruses that already have other mutations in PR. Alternatively, N88S mutants may arise early but require additional changes in order to overcome fitness constraints before becoming a predominant quasispecies.

Based on HIV-1 PR crystal structure data, residue 88 is not directly involved in substrate or inhibitor binding (7). In wild-type PR structures, the side chain of N88 forms hydrogen bonds with the main-chain amide of T74, the main-chain carbonyl of T31, and the side-chain Oγ1 hydroxyl of T31 (Fig. 3). Mutation of N88 to a serine residue introduces a smaller side chain. In the published structure of the HIV-1 PR dimer complexed with amprenavir (Protein Data Bank entry 1HPV [7]; www.rcsb.org/pdb/), the D30 side chain of one PR subunit forms a hydrogen bond with the bound drug, and V32 forms part of a hydrophobic patch in the drug-binding pocket. The N88S mutation could lead to the formation of a hydrogen bond between S88 Oγ and the main-chain amide of T31, potentially

pulling the D30-T31 region towards the site of mutation. This potential change in the binding pocket could allow the aromatic ring of amprenavir to slide further in toward the protein surface, improving its hydrophobic interactions with amino acid residues such as A28, V32, I47, I50, and I84 (Fig. 3). Overall, these changes could improve the amprenavir binding, which would be consistent with the observed hypersensitivity of N88S HIV-1 PR to amprenavir. Biochemical characterization will be required to elucidate the underlying mechanism of drug susceptibility.

The intricate drug resistance phenotypes displayed by the N88S (in PR) and M184V (in RT) mutants may be paradigms for other potentially clinically relevant manifestations. The complexities of the resistance phenotypes could not have been accurately predicted by genotypic analysis alone. In light of the increasing number of patients undergoing highly active antiretroviral therapy, these results suggest that phenotypic testing is helpful in unraveling the potential synergistic interplay of multiple mutations in determining drug efficacy in vivo. The detection of drug hypersensitivity suggests a strategy for therapy management that can be tested in the context of a clinical trial.

We conclude from our studies that amprenavir may have increased clinical efficacy against N88S viruses. Previous clinical studies found that 3TC-zidovudine combination therapy was more beneficial than zidovudine monotherapy for patients harboring M184V viruses (8). By analogy, dual PRI therapy combining amprenavir with nelfinavir, indinavir, or new drugs such as BMS-232632 could delay the emergence of viruses resistant to both PRIs. Some such combinations have already been reported to be well tolerated (J. Eron et al., Abstr. 5th Conf. Retroviruses Opportunistic Infect., abstr. 6, 1998).

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REFERENCES

1. **Adachi, A., H. E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, and M. A. Martin.** 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J. Virol. 59:284–291.
2. **Boden, D., A. Hurley, L. Zhang, Y. Cao, Y. Guo, M. Duran, J. Tsay, J. Ip, C. Farthing, K. Limoli, N. Parkin, and M. Markowitz.** 1999. HIV-1 drug resistance in newly infected individuals. JAMA 282:1135–1141.
3. **Carpenter, C. C. J., M. A. Fischl, S. M. Hammer, M. S. Hirsch, D. M. Jacobsen, D. A. Katzenstein, J. S. G. Montaner, D. D. Richman, M. S. Saag, R. T. Schooley, M. A. Thompson, S. Vella, P. G. Yeni, and P. A. Volberding.** 1998. Antiretroviral therapy for HIV infection in 1998. Updated recommendations of the International AIDS Society-USA panel. JAMA 280:78–86.
4. **Coffin, J. M.** 1995. HIV population dynamics in vivo: implications for genetic variation, pathogenesis, and therapy. Science 267:483–489.

4a. **Gong, Y.-F., B. S. Robinson, R. E. Rose, C. Deminie, T. P. Spicer, D. Stock, R. J. Colonno, and P.-F. Lin.** In vitro resistance profile of the human immunodeficiency virus type 1 protease inhibitor BMS-232632. Antimicrob. Agents Chemother., in press.

5. **Hecht, F. M., R. M. Grant, C. J. Petropoulos, B. Dillon, M. A. Chesney, H. Tian, N. S. Hellmann, N. I. Bandrapalli, L. Digilio, B. Branson, and J. O. Kahn.** 1998. Sexual transmission of an HIV-1 variant resistant to multiple reverse-transcriptase and protease inhibitors. N. Engl. J. Med. 339:307–311.
6. **Hirsch, M. S., B. Conway, R. T. D'Aquila, V. A. Johnson, F. Brun-Vezinet, B. Clotet, L. M. Demeter, S. M. Hammer, D. M. Jacobsen, D. R. Kuritzkes, C. Loveday, J. W. Mellors, S. Vella, and D. D. Richman.** 1998. Antiretroviral drug resistance testing in adults with HIV infection: implications for clinical management. JAMA 279:1984–1991.
7. **Kim, E. E., C. T. Baker, M. D. Dwyer, M. A. Murcko, B. G. Rao, R. D. Tung, and M. A. Navia.** 1995. Crystal structure of HIV-1 protease in complex with VX-478, a potent and orally bioavailable inhibitor of the enzyme. J. Am. Chem. Soc. 117:1181–1182.
8. **Larder, B. A., S. D. Kemp, and P. R. Harrigan.** 1995. Potential mechanism for sustained antiretroviral efficacy of AZT-3TC combination therapy. Science 269:696–699.
9. **Lazdins, J., J. Mestan, G. Goutte, M. Walker, G. Bold, H. Capraro, and T. Klimkait.** 1997. In vitro effect of α_1-acid glycoprotein on the anti-human immunodeficiency virus (HIV) activity of the protease inhibitor CGP 61755: a comparative study with other relevant HIV protease inhibitors. J. Infect. Dis. 175:1063–1070.
10. **Little, S., E. Daar, R. D'Aquila, P. Keiser, E. Connick, J. Whitcomb, N. Hellmann, C. Petropoulos, J. Pitt, E. Rosenberg, R. Koup, and D. Richman.** 1999. Reduced antiretroviral drug susceptibility among patients with primary HIV infection. JAMA 282:1142–1149.
11. **Livingston, D., S. Pazhanisamy, D. Porter, J. Partaledis, R. Tung, and G. Painter.** 1995. Weak binding of VX-478 to human plasma proteins and implications for anti-human immunodeficiency virus therapy. J. Infect. Dis. 172:1238–1245.
12. **Murphy, R., R. Gulick, V. DeGruttola, R. D'Aquila, J. Eron, J. Sommadossi, J. Currier, L. Smeaton, I. Frank, A. Caliendo, J. Gerber, R. Tung, and D. Kuritzkes.** 1999. Treatment with amprenavir alone or amprenavir with zidovudine and lamivudine in adults with human immunodeficiency virus infection. J. Infect. Dis. 179:808–816.
13. **Palmer, S., R. W. Shafer, and T. C. Merigan.** 1999. Highly drug-resistant HIV-1 clinical isolates are cross-resistant to many antiretroviral compounds in current clinical development. AIDS 13:661–667.
14. **Parkin, N., Y. Lie, N. Hellmann, M. Markowitz, S. Bonhoeffer, D. Ho, and C. Petropoulos.** 1999. Phenotypic changes in drug susceptibility associated with failure of human immunodeficiency virus type 1 (HIV-1) triple combination therapy. J. Infect. Dis. 180:865–870.
15. **Partaledis, J. A., K. Yamaguchi, M. Tisdale, E. E. Blair, C. Falcione, B. Maschera, R. E. Myers, S. Pazhanisamy, O. Futer, A. B. Cullinan, C. M. Stuver, R. A. Byrn, and D. J. Livingston.** 1995. In vitro selection and characterization of human immunodeficiency virus type 1 (HIV-1) isolates with reduced sensitivity to hydroxyethylamino sulfonamide inhibitors of HIV-1 aspartyl protease. J. Virol. 69:5228–5235.
16. **Patick, A. K., M. Duran, Y. Cao, D. Shugarts, M. R. Keller, E. Mazabel, M. Knowles, S. Chapman, D. R. Kuritzkes, and M. Markowitz.** 1998. Genotypic and phenotypic characterization of human immunodeficiency virus type 1 variants isolated from patients treated with the protease inhibitor nelfinavir. Antimicrob. Agents Chemother. 42:2637–2644.

16a. **Petropoulos, C. J., N. T. Parkin, K. L. Limoli, Y. S. Lie, M. T. Wrin, W. Huang, H. Tian, D. Smith, G. A. Winslow, D. Capon, and J. Whitcomb.** 2000. A novel phenotypic drug susceptibility assay for human immunodeficiency virus type 1. Antimicrob. Agents Chemother. 44:920–928.

17. **Sadler, B. M., C. D. Hanson, G. E. Chittick, W. T. Symonds, and N. S. Roskell.** 1999. Safety and pharmacokinetics of amprenavir (141W94), a human immunodeficiency virus (HIV) type 1 protease inhibitor, following oral administration of single doses to HIV-infected adults. Antimicrob. Agents Chemother. 43:1686–1692.
18. **Sarkar, G., and S. S. Sommer.** 1990. The "megaprimer" method of site-directed mutagenesis. BioTechniques 8:404–407.
19. **Schinazi, R. F., B. A. Larder, and J. W. Mellors.** 1999. Mutations in retroviral genes associated with drug resistance. Int. Antivir. News 7:46–69.
20. **Smidt, M. L., K. E. Potts, S. P. Tucker, L. Blystone, T. R. Stiebel, Jr., W. C. Stallings, J. J. McDonald, D. Pillay, D. D. Richman, and M. L. Bryant.** 1997. A mutation in human immunodeficiency virus type 1 protease at position 88, located outside the active site, confers resistance to the hydroxyethylurea inhibitor SC-55389A. Antimicrob. Agents Chemother. 41:515–522.
21. **St. Clair, M. H., J. Millard, J. Rooney, M. Tisdale, N. Parry, B. M. Sadler, M. R. Blum, and G. Painter.** 1996. In vitro antiviral activity of 141W94 (VX-478) in combination with other antiretroviral agents. Antivir. Res. 29: 53–56.
22. **Tucker, S. P., T. R. Stiebel, Jr., K. E. Potts, M. L. Smidt, and M. L. Bryant.** 1998. Estimate of the frequency of human immunodeficiency virus type 1 protease inhibitor resistance within unselected virus populations in vitro. Antimicrob. Agents Chemother. 42:478–480.